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Insulin stimulated glucose uptake in adipocytes requires the co-localization of myosin IIA (myoIIA) and glucose transporter 4 (GLUT4) at the plasma membrane. Our previous studies have shown that glucose uptake decreases dramatically if either GLUT4 or myoIIA are inhibited from translocating to the plasma membrane during insulin stimulated glucose uptake. Our research showed a co-localization pattern between myoIIA and filamentous actin (F-actin) at the plasma membrane upon insulin stimulation. In order to determine whether calmodulin and calcium regulate myoIIA and F-actin during insulin stimulated glucose uptake, we altered calcium and calmodulin levels within adipocytes and noticed a decrease in co-localization of GLUT4, myoIIA, and F-actin at the plasma membrane. Although our results did not establish what type of interaction myoIIA and F-actin have at the plasma membrane, our experiments did provide evidence that the two proteins colocalize, with GLUT4 at the plasma membrane. Furthermore, we established that calcium and calmodulin are important regulatory factors in the co-localization of GLUT4, myoIIA, and F-actin to the plasma membrane during insulin stimulated glucose uptake. In order to obtain high resolution images of the cell surface to more closely study the effects of insulin stimulation on GLUT4, myoIIA, and F-actin, we established a new protocol for cellular preparation of 3T3-L1 adipocytes for viewing under the helium ion microscope. With the helium ion microscope, cells can be viewed at a higher magnification than most microscopes. The protocols we established

for biological preparation will allow for future studies using helium ion microscopy, looking at distribution of target proteins as well as structural changes during insulin stimulated glucose uptake in adipocytes.

INVESTIGATION OF THE INTERACTION BETWEEN MYOSIN IIA AND
FILAMENTOUS ACTIN DURING INSULIN STIMULATED
GLUCOSE UPTAKE IN 3T3-L1 ADIPOCYTES

by

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Approved by

Committee Chair

Dedicated to my mother Kim and my father Rick

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of the
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CHAPTER I

INTRODUCTION

Type II diabetes mellitus is becoming an ever increasing problem in today's society, affecting well over 10% of the population in some ethnic groups. It is characterized by the elevation of blood glucose levels, with the inability to maintain glucose homeostasis. Normally, excess glucose in the blood is taken up by skeletal muscle and adipose tissue in order to maintain glucose homeostasis, and during these hyperglycemic conditions insulin is released by β -cells of the pancreas into the bloodstream. Long durations of hyperglycemic conditions can render adipose tissue unresponsive to insulin. Insulin resistance, a precursor to diabetes, includes such symptoms as increased blood pressure, elevated triglycerides, and weight gain [1, 22]. Understanding the molecular mechanisms that can be disrupted in the insulin signaling pathway during diabetes is critical for researchers interested in developing pharmaceuticals targets.

Insulin signaling

The binding of insulin to its tyrosine kinase receptor, located on the cell surface of skeletal muscle cells and adipocytes, stimulates the phosphoinositide 3-kinase (PI3K) signaling pathway, and with its various downstream targets it ultimately allows for

glucose uptake to occur. PI3K is responsible for activation of Akt, which translocates glucose transporter 4 (GLUT4) vesicles from the perinuclear region to the plasma membrane [12, 39]. GLUT4 is the glucose transporter stimulated by insulin in skeletal muscle cells and adipocytes. It is specifically this glucose transporter that allows for high levels of glucose uptake during hyperglycemic conditions [12]. Studies have shown the importance of PI3K and Akt in the insulin stimulated glucose uptake pathway, with inhibition of either preventing GLUT4 translocation to the plasma membrane. After fusion of GLUT4 with the plasma membrane, the transporter allows for the entrance of glucose in skeletal muscle cells as well as adipocytes [21-23, 25].

Cytoskeletal reorganization

Studies have shown that F-actin must undergo reorganization during insulin stimulated glucose uptake in order for GLUT4 translocation and fusion to occur. This is due to the fact that actin acts as a barrier between the cytosol and the plasma membrane [8, 34]. Insulin stimulation in adipocytes has been recognized as a catalyst for cytoskeletal reorganization. The underlying mechanisms regulating actin reorganization during insulin stimulated glucose uptake are still unknown. However, the motor protein myoIIA is thought to play a crucial role in assisting F-actin reorganization, and thus in GLUT4 fusion and glucose uptake as well [2, 3].

Non-muscle myosin II

Myosin II, a motor protein, is also important in insulin stimulated glucose uptake. Of the 15 different classes of myosin, myosin II is referred to as the conventional myosin. Described as a two-headed myosin, myosin II contains a pair of heavy chains (MHC), a

pair of essential light chains, and a pair of regulatory light chains (RLC). There are three isoforms of myosin II (A, B, C), differing in the heavy chain region. The globular head, located on the heavy chain, is the location on which actin and ATP bind, resulting in the motor activity of myosin [26, 27].

There are two types of myosin II; muscle and non-muscle. Non-muscle myosin II is very similar to that of muscle myosin II, with both types containing a phosphorylation site on the RLC to regulate myosin activity. However, a stark difference between non-muscle and muscle myosin II is the involvement of non-muscle myosin II in the cytoskeletal remodeling of F-actin [26, 27]. Insulin stimulation causes actin reorganization to occur within an adipocyte. Myosin II assists with actin reorganization, by either binding and contracting F-actin or depolymerizing F-actin [9,18], which is essential for the movement of PI3K to its downstream targets, such as Akt. This allows for translocation of GLUT4 along the actin tracks during insulin stimulation, ultimately allowing for GLUT4 fusion with the plasma membrane [24].

Role of myosin II during insulin stimulation

Myosin has a documented role in vesicle trafficking. The most characterized examples of actin-dependent movement of organelles have been observed in plant systems [38]. The movement of vesicles (such as GLUT4 vesicles) by myosin along actin tracks is also noted in adipocytes [4]. However, myosin II has been shown to have no effect on GLUT4 trafficking to the plasma membrane [4]. Instead, this motor protein has instead been implicated in GLUT4 docking and fusion with the plasma membrane [4].

The two myosin II isoforms present in adipocytes, myoIIA and myoIIB, have been found to have different localization patterns during insulin stimulation. Studies have shown that myoIIB is localized at the cell cortex and does not change its localization in the presence of insulin, whereas myoIIA will translocate from a perinuclear region to the plasma membrane in response to insulin. Insulin triggers phosphorylation of the regulatory light chain (RLC) associated with myoIIA via myosin light chain kinase (MLCK) [2, 21]. Activating myoIIA causes its movement and co-localization with GLUT4 at the plasma membrane. When both proteins colocalize at the plasma membrane, GLUT4 fusion occurs. The movement of myoIIA to the plasma membrane has been shown to be necessary for glucose uptake to occur. Studies inhibiting myoIIA have shown that GLUT4-mediated insulin stimulated glucose uptake did not occur effectively even when GLUT4 translocated to the plasma membrane. This is due to the lack of GLUT4 fusion with the membrane regardless of its placement under the plasma membrane. Thus, the presence of myoIIA at the plasma membrane is necessary for fusion of GLUT4 with the plasma membrane to occur [3,4].

Regulators of insulin stimulated glucose uptake

The phosphorylation of the RLC on myoIIA by MLCK is one activator of myoIIA, causing its translocation to the plasma membrane and ultimately allows for GLUT4 fusion with the plasma membrane. MLCK is a calcium/calmodulin kinase responsible for activating non-muscle myoIIA in adipocytes [35].

Studies have shown that the lack of intracellular calcium available for activation of MLCK will prevent GLUT4 fusion with the plasma membrane due to the inactivity of

myoIIA [4, 18, 30, 40]. However, calmodulin has been found to bind to MLCK without being a part of the calcium/calmodulin complex [36]. The calmodulin does not activate MLCK without calcium binding to the calmodulin. Calcium-calmodulin kinase II has also been found to require calcium/calmodulin modulation that will allow it to activate MLCK. This further demonstrates the importance of calcium and calmodulin in the activation of the MLCK pathway [37].

Actin remodeling

Studies have shown that cortical actin remodeling must occur in order for GLUT4 fusion with the plasma membrane [8]. Conventional muscle myosin II typically contributes to the contractile force coinciding with myosin-actin cross bridging, suggesting that non-muscle myoIIA's interaction with cortical F-actin filaments may contract F-actin, thus reorganizing cortical actin and allowing for GLUT4 fusion with the plasma membrane in adipocytes [23]. Studies have also shown that knocking down or inhibiting myosin II results in decreased glucose uptake, indicating that myosin II is necessary for glucose uptake [3]. GLUT4 still translocated to the membrane, but failed to fuse with the plasma membrane in the absence of myosin II [4]. Thus, what is not known is how myosin II assists in GLUT4 fusion. It has been suggested that myosin II will contract cortical actin to allow GLUT4 vesicles to fuse with the plasma membrane. [3, 4].

Helium ion microscope

To discover new methodologies and techniques for analyzing morphological and structural changes at the cell surface during insulin stimulation in adipocytes, we decided to explore helium ion microscopy (HIM). This microscope allows for extremely high

resolution images when compared to other microscopes, such as the gallium focused ion beam (gallium FIB) and scanning electron microscope (SEM). The HIM was created to alleviate some of the problems associated with the FIB and SEM [5].

The most noteworthy problem associated with the gallium FIB that is improved in the HIM deals with the size of the ion used in the microscope (gallium versus helium). The gallium ions are big enough to cause substantial sample damage if the beam is at high enough energy. An ion of this size also requires more energy than either an electron or helium ion to travel in a beam [6]. At low enough energy, where destruction is less likely to occur, gallium ions may penetrate samples up to 70 nm in depth, allowing for some detailed information about the shape and form of the sample. The point of entrance of the ion (noted as the excited volume) is relatively large, negatively affecting contrast. However, helium ions are able to penetrate well past 100 nm in depth and have a very narrow excited volume, giving much sharper images of the surface [5, 7].

The HIM improved on the issue in high powered microscopes in which the diffraction of electrons occurs when they are meant to liberate secondary electrons. Since electrons are relatively small they tend to backscatter during imaging, and this scatter activates secondary electrons that are not from the targeted area, and thus resolution decreases. Due to the mass of electrons, less energy is used in an electron beam versus a helium beam in order to maintain contrast. As a result, sharpness is lost from the loss of electrons that is due to a rate of diffraction that becomes higher as energy decreases. The helium ion has a large mass in comparison, and therefore diffraction of ions from the helium ion beam is more limited. Electrons do not penetrate to depths beyond 50 nm,

resulting in less detail of the sample whereas a helium ion can penetrate to depths of more than 100 nm. Electrons also have broad excited volumes, which limit information about depth and surface of the sample [5, 7]. Because the HIM offers higher resolution than the SEM and FIB, we created a protocol for biological sample preparation in order to view adipocyte cell surfaces in great detail. We based the preparation on previous protocols for the SEM and FIB, due to the similar nature of the microscopes.

CHAPTER II

MATERIALS AND METHODS

Materials

Delbecco's Modified Eagle Medium High Glucose (DMEM) 1X cat # 11995 was purchased from Gibco. Ten cm cell culture plates were purchased from Falcon, cat # 35-3002. Fifteen mm German RD circular coverslips were purchased from Belco, cat # 1943-00015. From Sigma-Aldrich we purchased insulin from bovine pancreas cat # 15500-1G 016K1248, dexamethasone and 3-isobutyl 1-methyl-xanthine (MIX), cat # I5879, secondary fluorescent rabbit antibody, cat # A6154, phalloidin for F-actin staining, cat # P2141, inhibitor W13, cat # 88519-57-7, and poly-d-lysine, cat# P6407 . GLUT4 primary antibody was purchased from Santa Cruz Biotechnology, Inc., cat # 1608. Primary antibody for myosin IIA was from Covance, cat # PRB-440-P. Inhibitors BAPTA-AM and A23187 were purchased from Invitrogen, cat #s B-6769 and A-1493 respectively.

Cell culture

3T3-L1 fibroblasts from the mouse preadipocyte cell line were grown to confluency in medium containing 10% Bovine Calf Serum (BCS) in High Glucose Dulbecco's Modified Eagle Media (DMEM). Upon reaching confluency, differentiation of the cells into adipocytes was induced by 10% Fetal Bovine Serum (FBS) in High

Glucose DMEM with 0.52 mM 3-isobutyl 1-methyl-xanthine (MIX), 1.7 μ M insulin, and 1 μ M dexamethasone (DEX). After two days (Day 2), cells were incubated in 10% FBS in high glucose DMEM with 0.425 μ M insulin. Following this second treatment, media was changed with 10% FBS in high glucose DMEM every 48 hours [20].

Confocal microscopy

3T3-L1 cells were cultured and differentiated on glass coverslips. Between Day 7 and 9 the cells were serum starved for 4 hr and then treated with one of the following: 100 nM insulin for 15 min, 50 μ M BAPTA-AM in MeSO for 15 min followed by 100 nM insulin for 15 min, 0.1 μ M A23187 in MeOH for 10 min, or 70 μ M W-13 in dH₂O for 15 min followed by only 100 nM insulin for 15 min. Cells were then fixed with 4% formaldehyde for 15 min and permeabilized with 0.1% triton X-100 for 10 min. Primary antibodies for myoIIA and GLUT4 were then incubated with the cells for one hour. Fluorescent phalloidin was used to stain for F-actin. Secondary anti-rabbit antibody was added to the myoIIA and GLUT4 antibodies for visualization via confocal fluorescence microscopy. Cells were washed three times with 1X phosphate buffered saline (PBS) for 5 min per wash between antibody incubations. Finally, the coverslips were mounted on a glass slide with 50% glycerol and sealed.

Quantifying protein distribution along the plasma membrane

GLUT4, myoIIA, and F-actin amount were quantified via intensity of fluorescence both cortically and within the cytoplasm. Intensity of proteins at the cortex and the cytoplasm were measured and the ratio of cortical/cytoplasmic signal was averaged for individual cells. From these two values, a ratio of cortical / cytoplasm was

taken for a number of cells under various conditions. Then, the ratios were added and an average for these ratios was obtained. We used a percent change formula, $[(\text{Treatment} - \text{Basal}) / (\text{Basal}) \times 100\%]$, to obtain a value observing the increase or decrease in protein localization at the plasma membrane.

Substrate optimization

3T3-L1 preadipocytes were cultured on several substrates. These included glass coverslips, aluminum washers, aluminum foil wrapped around aluminum washers, and gold coated coverslips.

To coat coverslips in gold we used a gold sputter coater, which is normally used in SEM preparation. The sputter coater was kept on for duration of 60-90 seconds during the sputtering process, and the voltage was kept at a constant 10 mA.

Cell sample preparation optimization

Using day 7 differentiated adipocytes grown on gold coated coverslips, we washed the cells with 0.1M cacodylate buffer, pH 7.3, and fixed the cells in 2.5% glutaraldehyde, 2% formaldehyde in 0.1M cacodylate buffer, pH 7.3 for 30 min or 2 hr. Next, coverslips were washed with deionized water to avoid contamination of sample with salts from buffer solutions such as PBS. Optionally, we next permeabilized the adipocytes with 0.1% triton X-100 for 15 min or 1 hr. Following the wash, we used a secondary fixation of 4% osmium tetroxide for 30 min or 2 hours. Again, we washed the cells in deionized water. Next, we ran a dehydration series using acetonitrile, at the following concentrations: 25%, 50%, 75%, 95%, and 100% twice at 5 min each with the second 100% remaining on the sample overnight to evaporate. The next day, we attached

the coverslip to a piece of adhesive carbon tape and then the carbon tape to a microscope stub.

Plasma lawn assay for HIM

Between day 7 to 12 3T3-L1 adipocytes were serum starved for 4 hr. We treated cells with or without 100 nM insulin. After 15 min, we washed the coverslips with 0.1M cacodylate buffer, pH 7.3. Cells were fixed in 4% formaldehyde, 2% glutaraldehyde in deionized water for 45 min. We then rinsed cells in deionized water. For 1 min, the cells were incubated in poly-d-lysine at 0.5 mg/mL. The cells were rinsed three times in hypotonic buffer, containing 23 mM KCl, 10 mM Hepes, 2 mM MgCl₂, and 1mM EDTA, pH 7.5. Next, we added sonication buffer (70 mM KCl, 30 mM Hepes, 6 mM MgCl₂, 3 mM EGTA, 1 mM dithrothreitol, 0.1 mM phenylmethylsulphonyl fluoride, pH 7.5) to the plate. The coverslips were sonicated at power 4-5 with a 5 second pulse. The plates were washed with deionized water to remove salts. Next, we performed a dehydration series with acetonitrile at 25%, 50%, 75%, 95%, 100% (5 min each) and a final 100% overnight duration. The coverslips were then attached to microscopy stubs via sticky carbon tape.

HIM sample preparation for days of differentiation in adipocytes

The fixing process described next was repeated for the following days of differentiation: two days beyond visual confluence of preadipocytes and day 0, day 2, day 4, and day 7 adipocytes.

We washed the cells in 0.1M cacodylate buffer, pH 7.3. Then the cells were fixed overnight in 2.5% glutaraldehyde, 2% formaldehyde in 0.1M cacodylate buffer at pH 7.3.

The coverslips were washed with deionized water. We then treated the cells with 0.1% triton x-100 for 15 min, followed by a wash with deionized water. The cells were then secondarily fixed with 4% osmium tetroxide for 1 hour. Finally, we washed the cells with deionized water, followed by a dehydration series in acetonitrile: 25%, 50%, 75%, 95%, 100% (5 min each) and a final 100% overnight duration.

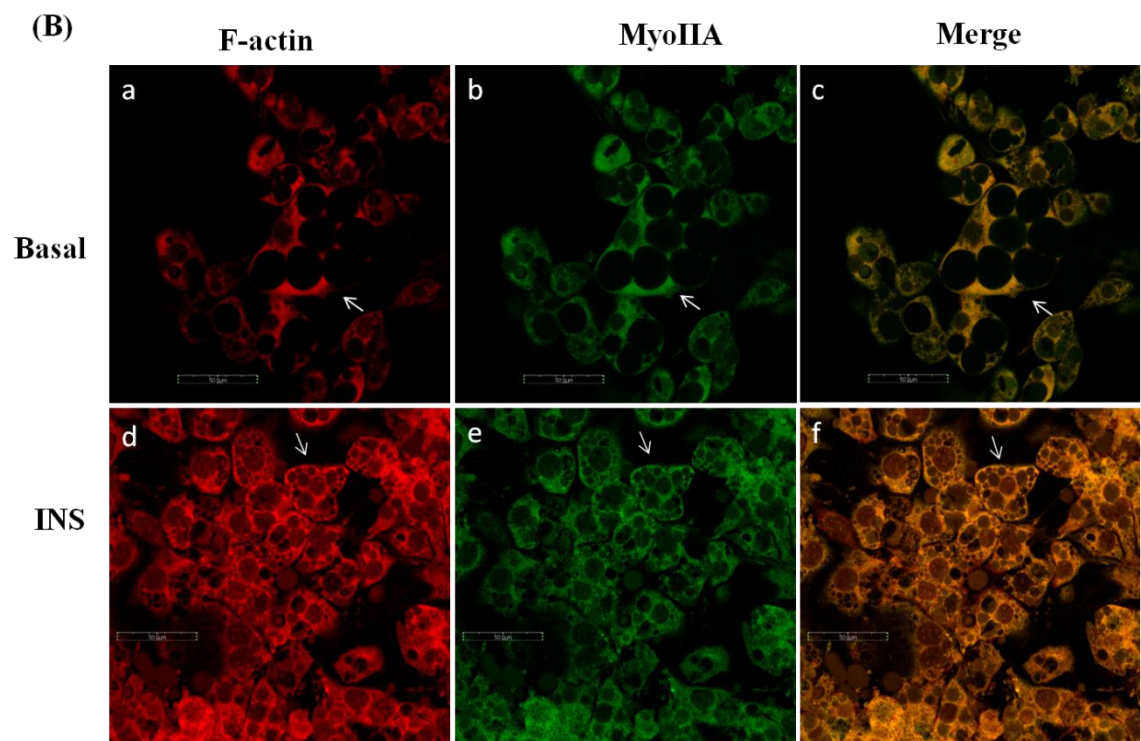
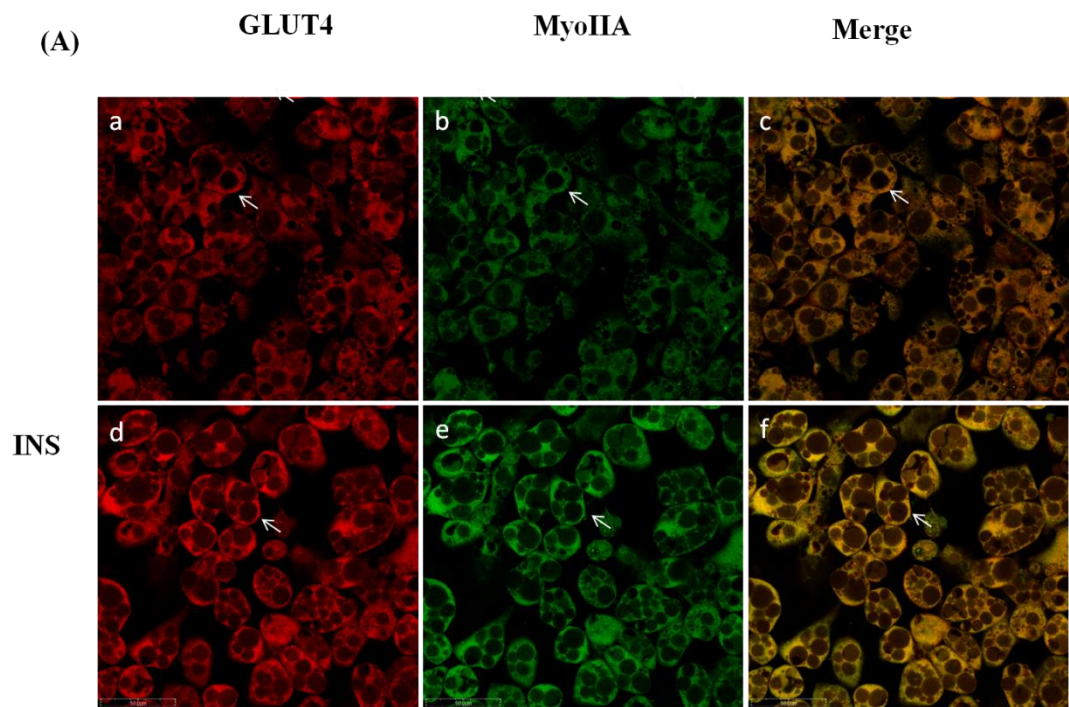
CHAPTER III

RESULTS

MyoIIA and F-actin colocalize at the plasma membrane during insulin stimulated glucose uptake

Previous studies have shown that in order for insulin stimulated glucose uptake to occur, myoIIA and GLUT4 localization and remodeling of F-actin at the plasma membrane is required [3, 4, 8]. Although studies have shown that myoIIA and GLUT4 co-localization allows for GLUT4 docking and fusion with the plasma membrane [2], the necessity of F-actin and myoIIA co-localization has not been determined. However, studies *in vitro* using myoIIA and F-actin filaments have shown a link for co-localization during the redistribution process of F-actin remodeling. Evidence obtained from these experiments demonstrates that myosin II controls the turnover rate of actin bundles [9]. Myosin II and F-actin have also been found to have several functions in non-muscle cells, assisting in the processes of mitotic spindle assembly, cell spreading, and cell migration [28]. Because actin and myosin filaments normally overlap in muscle cells to allow for the contraction of actin, a pattern of co-localization of these two proteins at the plasma membrane would provide evidence toward a similar occurrence in 3T3-L1 adipocytes during insulin stimulated glucose uptake.

In order to determine if these two key players interact, we first needed to establish if myoIIA and F-actin colocalize at the plasma membrane upon insulin stimulation. To do this, we serum starved fully differentiated day 7 3T3-L1 adipocytes for 4 hr, followed by treatment in the presence or absence of insulin. Following treatment, cells were fixed in formaldehyde and then incubated in primary antibody for GLUT4 and myoIIA, or the phallotoxin phalloidin for F-actin staining. Fluorescent secondary antibodies were then used to label GLUT4 and myoIIA for confocal microscopy. As expected, GLUT4 and myoIIA colocalized at the plasma membrane in the presence of insulin (Fig 1A). Visualization under the confocal microscope indicated a clear insulin-dependent co-localization of myoIIA and F-actin at the plasma membrane (Fig 1B). Localization of the three proteins at the plasma membrane increased by 30-90% once stimulated with insulin (Fig 1C). These results support the evidence that myoIIA -F-actin co-localization occurs in proximity to GLUT4 docking and fusion with the plasma membrane [2].



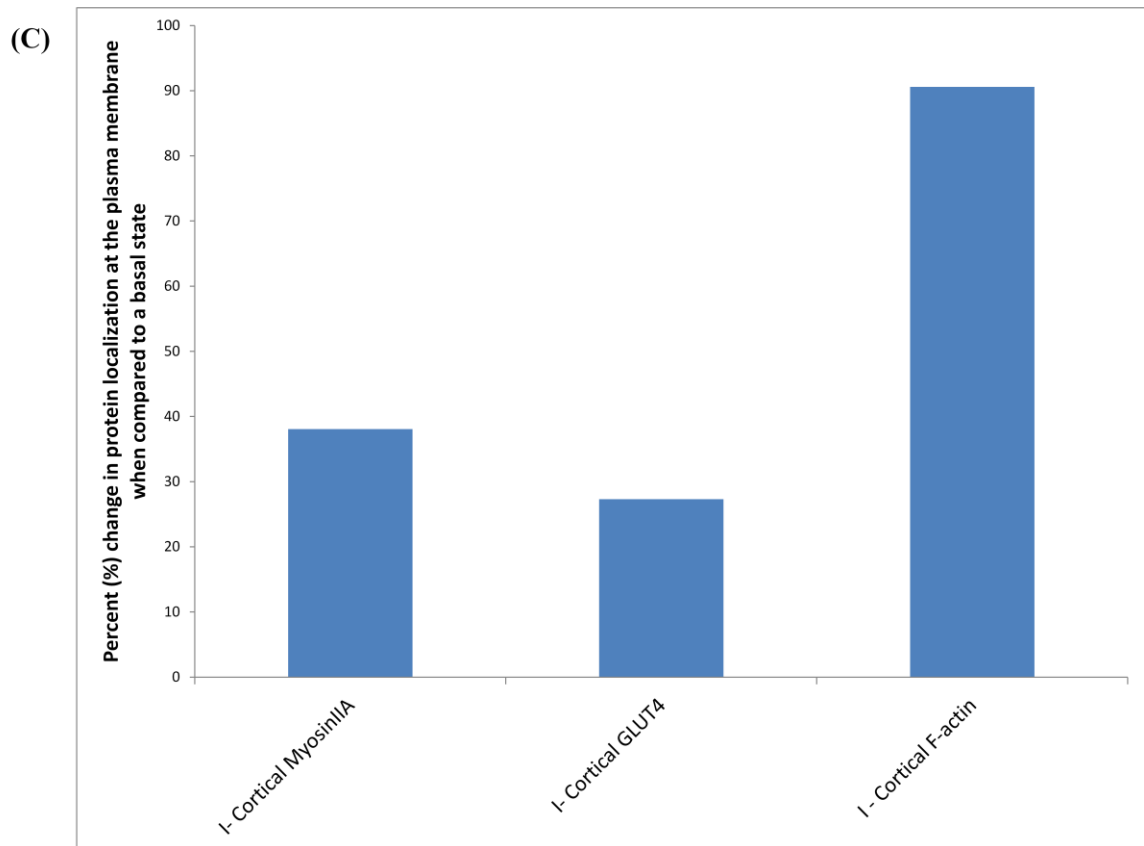
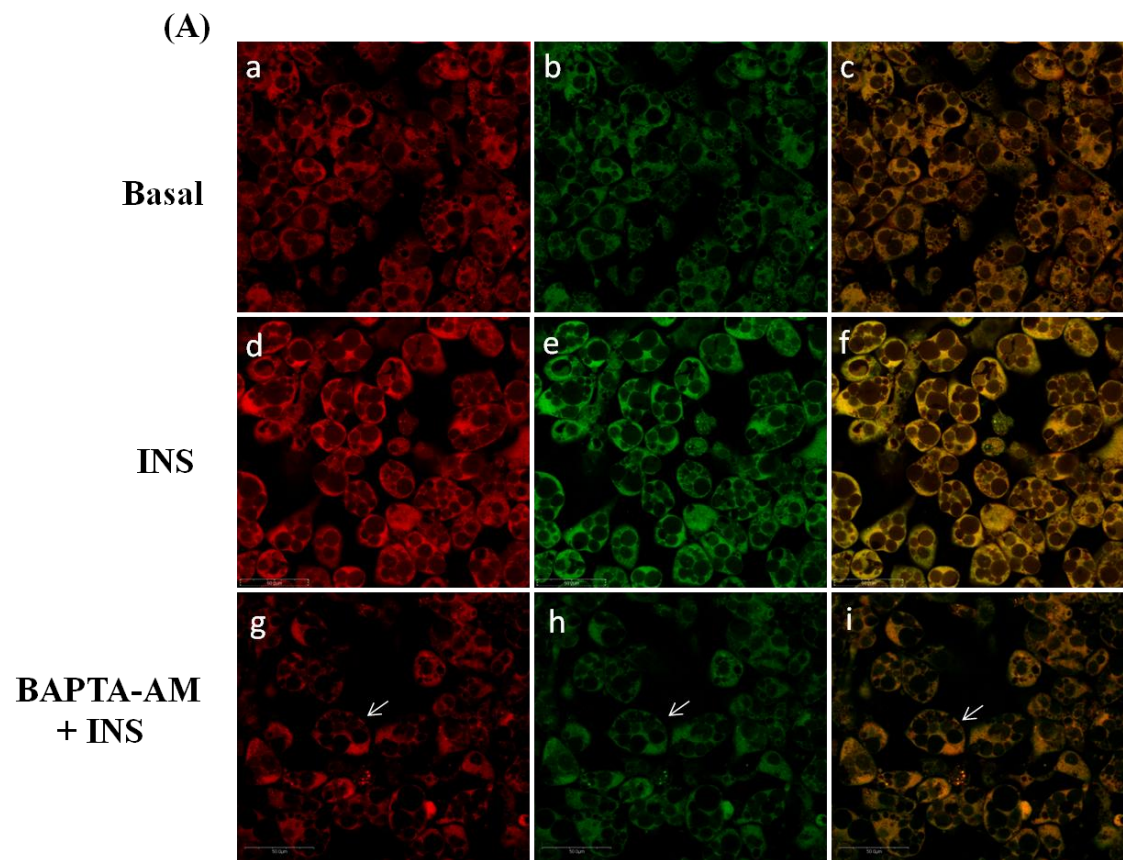
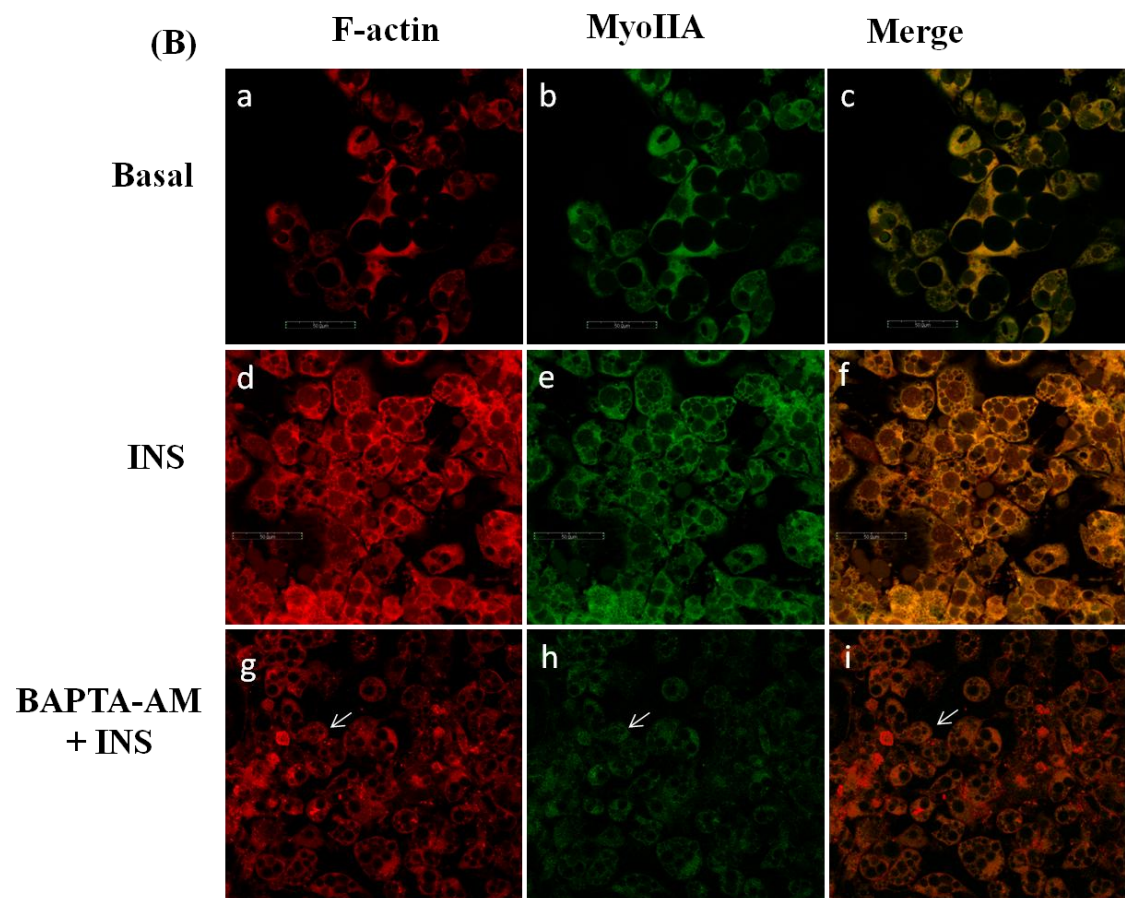


Figure 1. GLUT4, myoIIA, and F-actin localize to the plasma membrane in response to insulin. (A), (B) 3T3-L1 day 7 adipocytes were serum starved for 4 hr and then either left untreated (basal) or treated with 100 nM insulin for 15 min. Specific antibodies were used for confocal microscopy to determine the localization of GLUT4, myoIIA, and F-actin, as described in the materials and methods section. (C) Distribution of GLUT4, myoIIA, or F-actin within the cell and at the plasma membrane under basal and insulin conditions was quantified as described in material and methods. Briefly, ratios of intensity comparing the cytoplasm to the plasma membrane were taken, and then a percent difference of basal to insulin was calculated. Arrows demonstrate that there is a difference in the amount of protein located at the plasma membrane between the insulin treatments and basal. Images are representative of 3 independent experiments.

Calcium and calmodulin are required for myoIIA and F-actin co-localization at the plasma membrane

Studies observing co-localization patterns of myoIIA and F-actin stress the importance of the role of calcium in the actin polymerization and reorganization necessary for secretory granules to be able to dock with the plasma membrane [29]. Those studies also suggest that the same process may occur in adipocytes to allow for GLUT4 docking and fusion with the plasma membrane during insulin stimulation. Because calcium and calmodulin are involved in pathways that activate MLCK, myoIIA, GLUT4, and F-actin, our studies used the inhibitor BAPTA-AM to chelate calcium during insulin stimulation to determine how alterations in calcium levels affects GLUT4, myoIIA and F-actin translocation and configuration at the plasma membrane. To visualize the localization of GLUT4, myoIIA, and F-actin, we used confocal microscopy (Figs 2A, 2B). Previous results from our lab have shown that the movement of both GLUT4 and myoIIA to the plasma membrane is crucial for glucose uptake to occur [2]. With BAPTA-AM treatment, our results show that the amount of localization for GLUT4 and myoIIA remain near basal levels while F-actin localization was less than half that of insulin stimulation, indicating the necessity of calcium in the co-localization of GLUT4, myoIIA, and F-actin at the plasma membrane (Fig 2C).





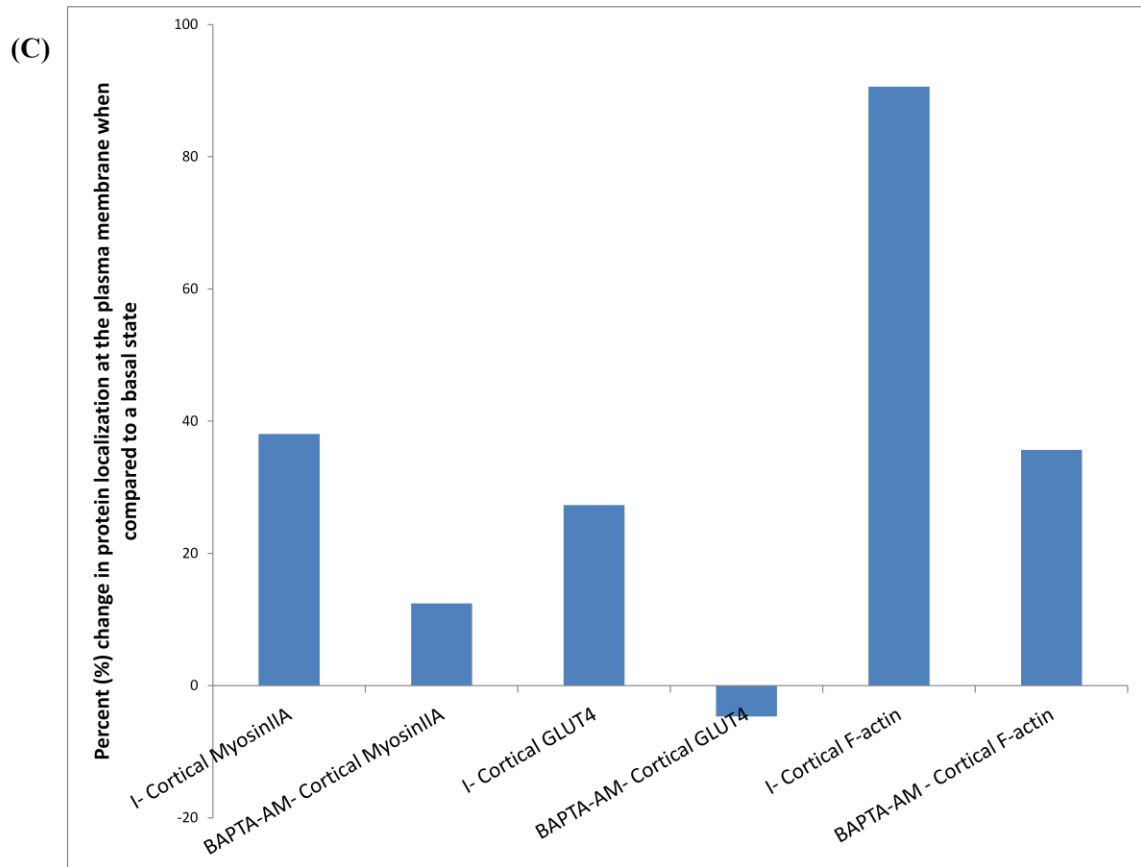


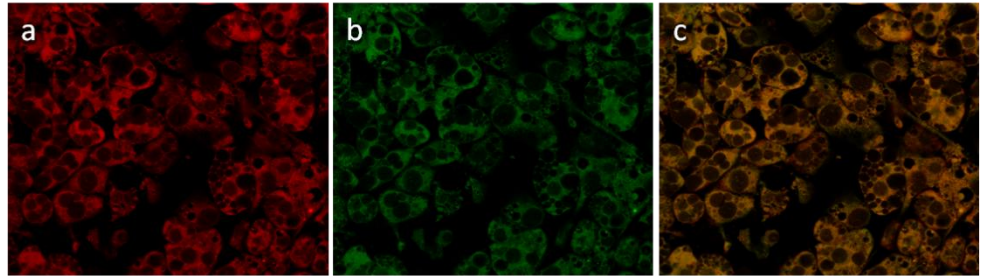
Figure 2. BAPTA-AM prevented translocation of GLUT4, myoIIA, and F-actin to the plasma membrane during insulin stimulation. (A), (B) 3T3-L1 day 7 adipocytes were serum starved for 4 hr and then were left untreated (basal), treated with 100 nM insulin for 15 min, or treated with 50 μ M BAPTA-AM in MeSO for 15 min followed by 100 nM insulin for 15 min. Antibodies were used to determine the localization of GLUT4, myoIIA, and F-actin via confocal microscopy, as described in the materials and methods section. (C) Distribution of GLUT4, myoIIA, and F-actin localized at the plasma membrane was quantified as is described in the materials and methods section. Briefly, ratios of intensity comparing the cytoplasm to the plasma membrane were taken, and then a percent difference of basal to insulin was calculated. The arrows are indicative that protein levels at the plasma membrane decreased with BAPTA-AM treatment. Images are representative of 3 independent experiments.

Serum starved adipocytes were subjected next to the ionophore A23187 to allow for increased intracellular calcium levels, in order to activate MLCK in the absence of insulin. The cells were incubated with primary and fluorescent secondary antibodies, as

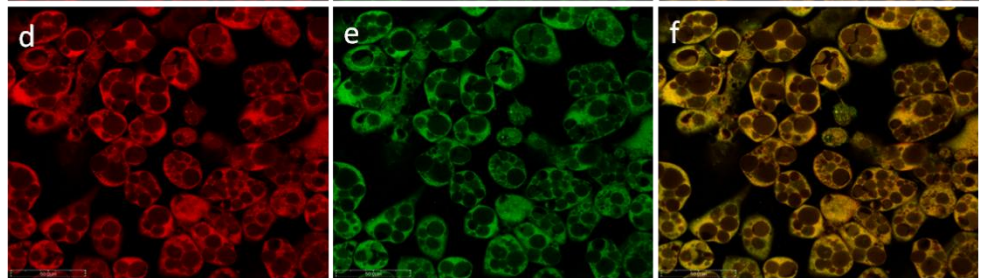
well as phalloidin for visualization of GLUT4, myoIIA, and F-actin by confocal microscopy (Figs 3A, 3B). Our results indicated that the proteins GLUT4 and F-actin had no significant change in translocation, maintaining near basal levels. However, myoIIA translocation and localization reached levels under ionophoric conditions greater than when treated with only insulin (Fig 3C). Therefore, we determined that calcium alone was not sufficient to induce the translocation of GLUT4 and F-actin, but was sufficient for inducing translocation of myoIIA to the plasma membrane in adipocytes.

(A)

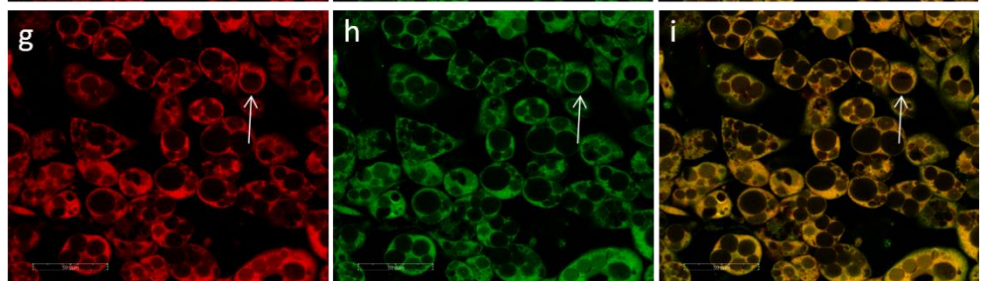
Basal

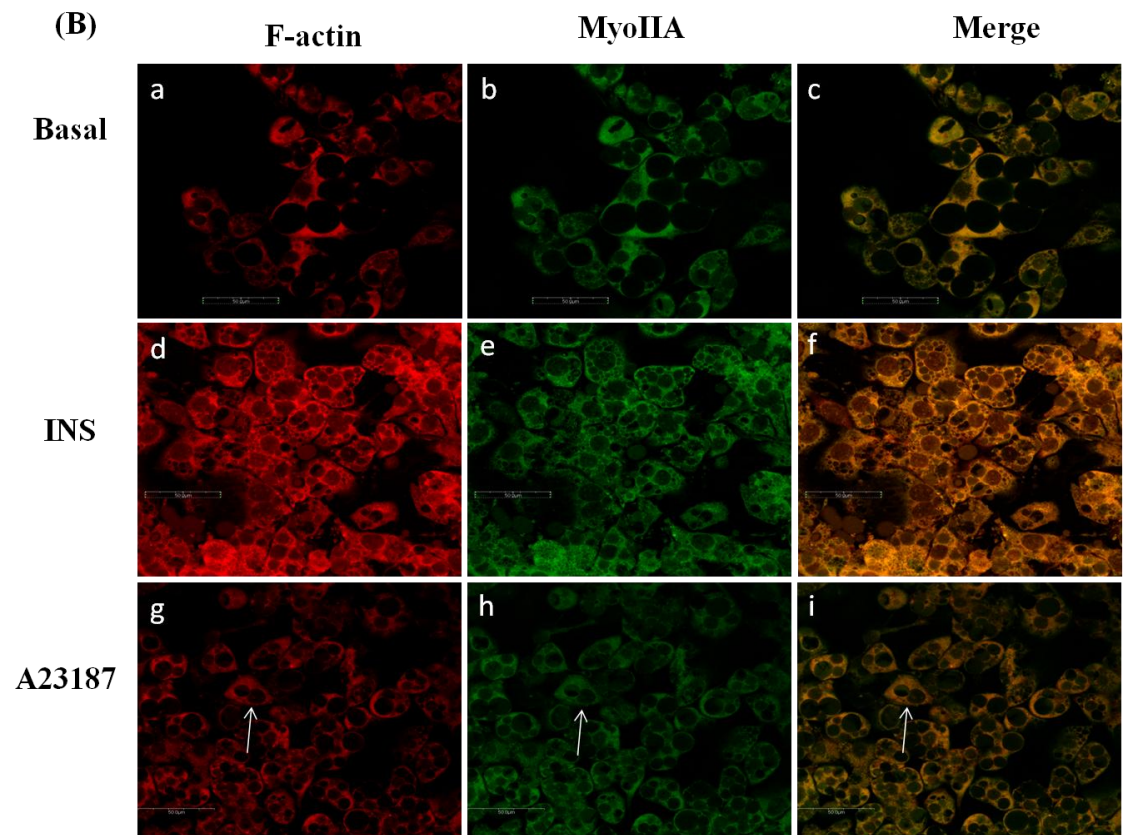


INS



A23187





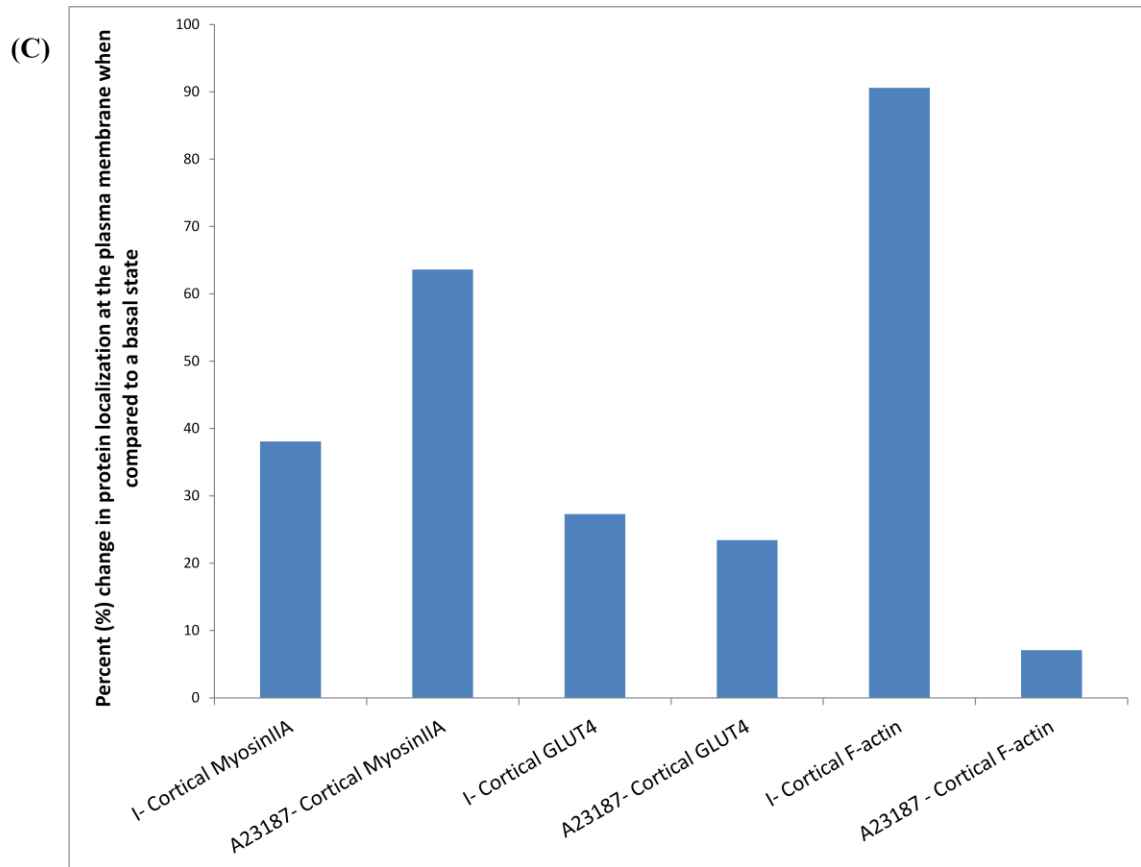
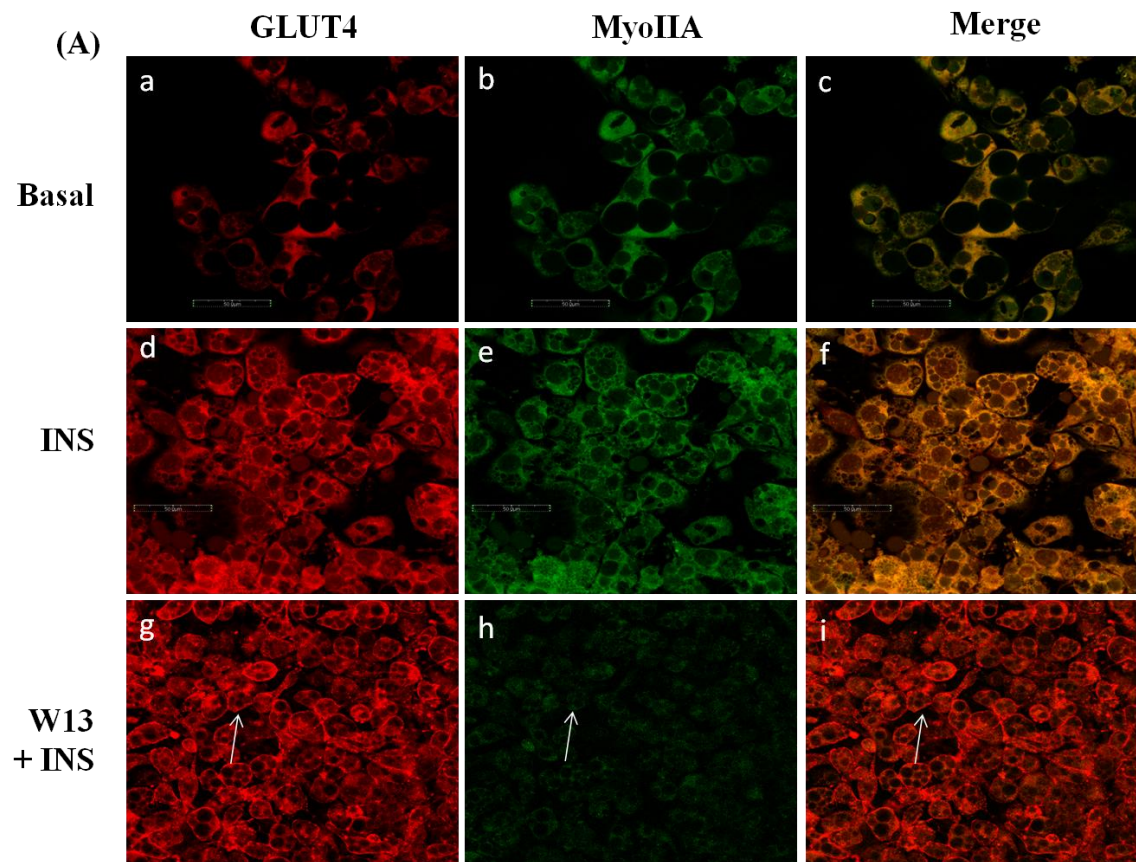
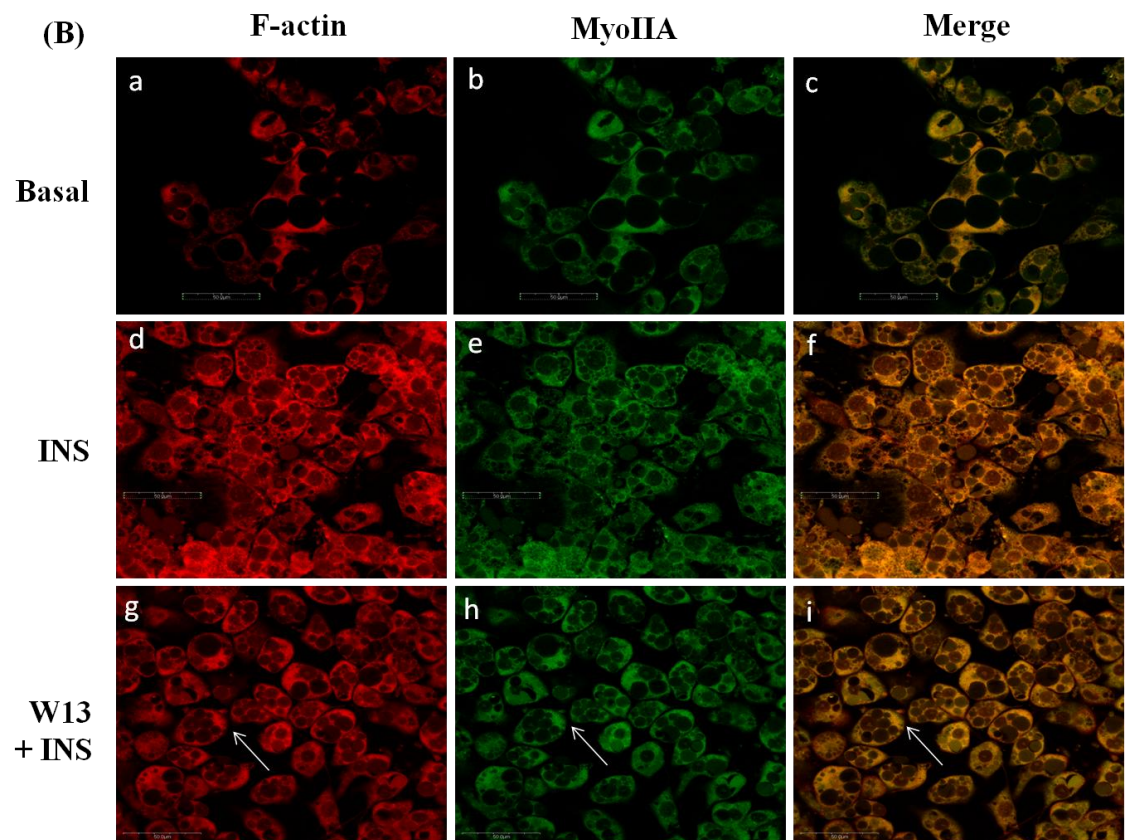


Figure 3. An increase in intracellular calcium alone was not sufficient to cause translocation of GLUT4, myoIIA, or F-actin in the absence of insulin. (A), (B) 3T3-L1 day 7 adipocytes were serum starved for 4 hr and then either left without treatment (basal), treated with 100 nM insulin for 15 min, or treated with 0.1 μ M A23187 in MeOH for 10 min. Confocal microscopy was used for visualization of localization for GLUT4, myoIIA, and F-actin. (C) Distribution of GLUT4, myoIIA, and F-actin within the cell and at the plasma membrane under basal, insulin or A23187 treated conditions was quantified as described in the materials and methods. Briefly, ratios of intensity comparing the cytoplasm to the plasma membrane were taken, and then a percent difference of basal to insulin was calculated. The arrows indicate localized proteins at the plasma membrane. Images are representative of 3 independent experiments.

We next used a calmodulin antagonist, W13, to prevent downstream effects that calcium has when forming a calcium-calmodulin complex. This complex activates MLCK, allowing it to phosphorylate myoIIA. Phosphorylation of myoIIA allows for its translocation to the plasma membrane [18]. Although not an activator of MLCK by itself, calcium-free calmodulin can become bound to MLCK before insulin stimulation occurs [30]. Calcium released into the system during insulin stimulation can then bind to these calcium-free calmodulin proteins that are attached to MLCK, activating the kinase.

Day 7 adipocytes were serum starved and treated with W13 followed by insulin stimulation. After incubation with primary and secondary antibodies targeting GLUT4 and myoIIA, as well as incubation with phalloidin for F-actin, we visualized the adipocytes via confocal microscopy (Figs 4A, 4B). As expected, myoIIA was inhibited from localizing to the plasma membrane when calmodulin was inactivated. Also, GLUT4 translocation was inhibited by the interaction of W13 with calmodulin. Finally, the reorganization of F-actin at the plasma membrane decreased by half when compared with insulin stimulation alone (Fig 4C).





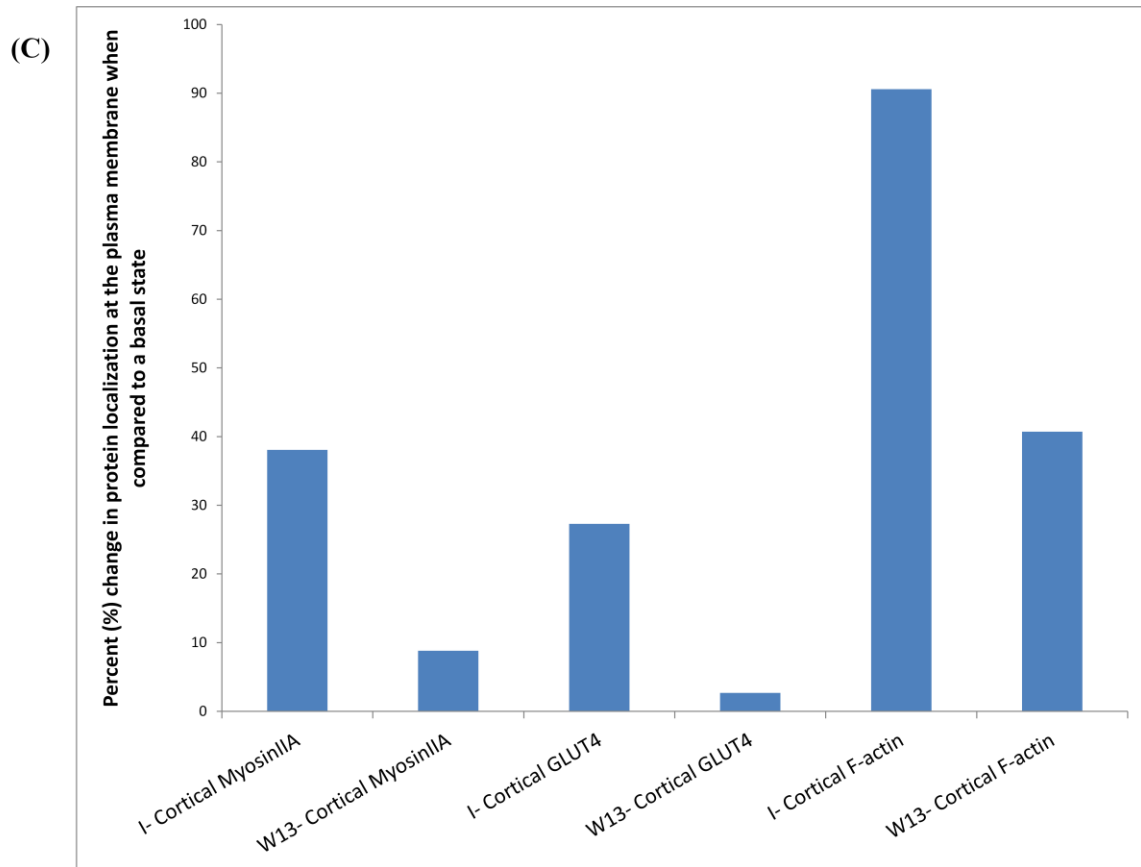


Figure 4. The calmodulin inhibitor W13 prevented translocation of GLUT4, myoIIA and F-actin. (A), (B) 3T3-L1 day 7 adipocytes grown on coverslips were serum starved for 4 hr and then either left untreated (basal), treated with 100 nM insulin for 15 min, or treated with 70 μ M W13 in H₂O for 15 min followed by 100 nM insulin for 15 min. Confocal microscopy was used for the visualization of localization of GLUT4, myoIIA, and F-actin (C) Distribution of GLUT4, myoIIA, and F-actin within the cell and at the plasma membrane under basal, insulin or W13 with insulin conditions was quantified as described in the materials and methods. Briefly, ratios of intensity comparing the cytoplasm to the plasma membrane were taken, and then a percent difference of basal to insulin was calculated. Arrows indicate localization of GLUT4, F-actin, and myoIIA at the plasma membrane. Images are representative of 3 independent experiments.

These studies indicated the necessity of calcium and calmodulin [30], for co-localization of GLUT4, myoIIA, and F-actin at the plasma membrane during insulin stimulation.

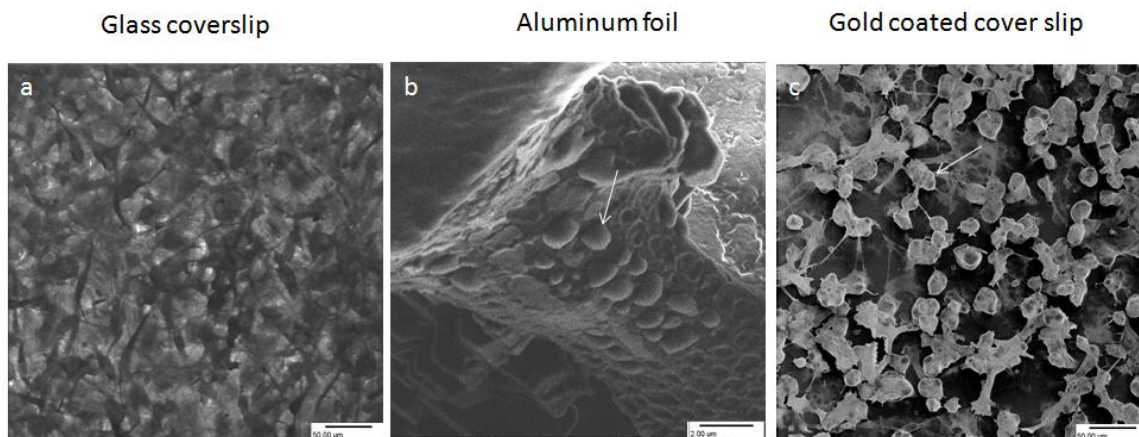


Figure 5. 3T3-L1 adipocytes grown on various substrates. Adipocytes were fixed overnight in 2% formaldehyde and 2.5% glutaraldehyde in 0.1M cacodylate buffer, 7.3 pH on Day 7 after differentiation was induced. Following that, acetonitrile was used to dehydrate the cell sample. (A) Adipocytes cultured on glass coverslips, (B) aluminum foil and (C) a glass coverslip layered with gold particles. Arrows indicate in (B) biological material from an adipocyte, and (C) adipocytes located on the gold coated coverslip.

Optimization of substrate for biological samples for using HIM

To further investigate the cell surface of adipocytes and the changes that occur during insulin stimulation, we ventured into high powered microscopy. The HIM showed potential as a platform to perform such experiments due to its improvements upon other microscopy. Previous studies have shown that HIM increases resolution by decreasing the amount of diffraction by secondary electrons produced by other types of microscopy such as SEM [6, 7]. The decrease in diffracting electrons from the sample is a technique that allows for higher resolution. With using a glass coverslip, we have the issue of lower

contrast due to the high level of diffracting electrons (Fig 5A). Since metals are known to be electron acceptors, culturing cells on a metal substrate is ideal. We tested the general viability of 3T3-L1 preadipocytes, as well as the ability to differentiate preadipocytes on various metallic substrates. We attempted to culture cells on flat, round aluminum washers as a result. Because the substrate is a solid, visualization of the cells was impossible using a light microscope. This affected the accuracy in the timing for inducing differentiation as described in the Materials and Methods section. Furthermore, cells were not detected on the aluminum washers when viewed under the HIM, indicating that the coating of the washers were either lethal to the cells or a poor substrate for cells to adhere to.

The next substrate we examined for cell culture was aluminum foil wrapped around the previously used aluminum washers. Because the foil is also solid, we again could not visualize preadipocytes during the culture phase. However, using the aluminum foil did solve the issue we had of cell viability or adherence. After preparing the sample as we did with the aluminum washers, cells were detected on the aluminum foil (Fig 5B). The contour of the aluminum foil was uneven, however, making it difficult to detect cells.

To try to address the issues of visibility and viability of our preadipocytes we coated glass coverslips with a thin layer of gold using a sputter coater. With a thin layer of gold as a substrate, we were able to observe cells with a light microscope and thus determine the proper timing for inducing differentiation into adipocytes. Also, there seemed to be no detrimental effect towards cell viability or differentiation, allowing for

visualization of cells with the HIM (Fig 5C). After fixing the cells in a formaldehyde/glutaraldehyde mixture, we dehydrated with acetonitrile. One artifact that we noticed from using gold was caused by particles sporadically detaching from the coverslip during the two weeks of incubation, resulting in some gold particles resting on top of the sample. We then compared the various substrates we used in order to determine the best substrate for preadipocyte and adipocyte cell culture.

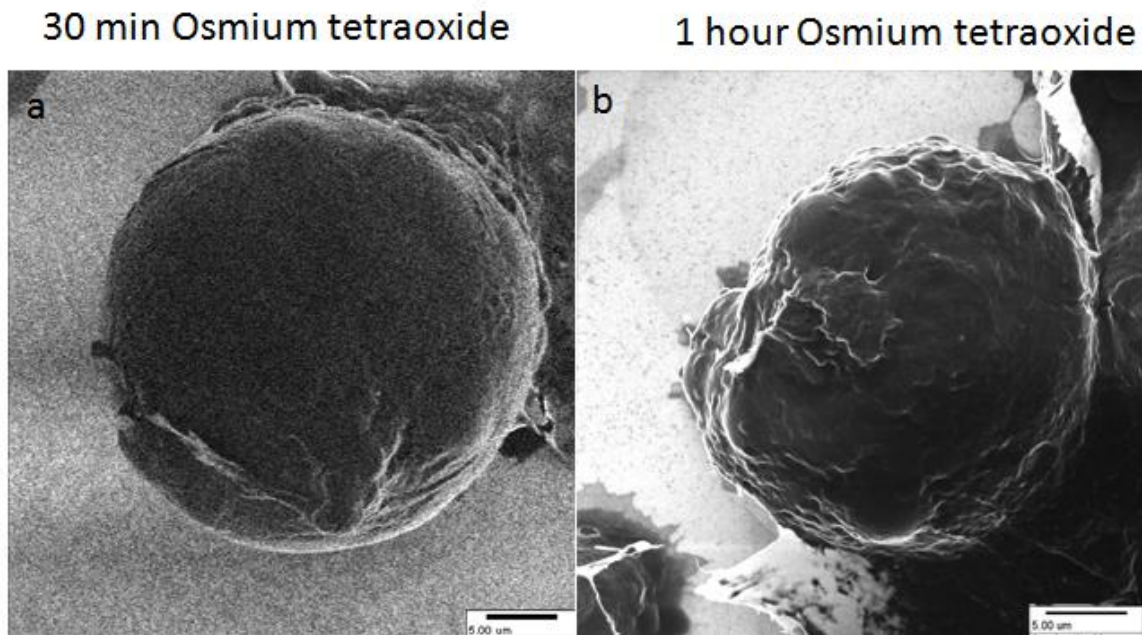


Figure 6. Osmium tetroxide fixation is more effective at 1 hr. Adipocytes were fixed overnight at Day 7 in 2% formaldehyde, 2.5% glutaraldehyde in 0.1M cacodylate buffer, 7.3 pH. Cells were then subjected to 4% osmium tetroxide for various durations. A dehydration series using acetonitrile followed. **(A)** Adipocytes fixed in 4% osmium tetroxide for 30 min and **(B)** adipocytes fixed in 4% osmium tetroxide for 1 hour.

Optimization of fixative for biological samples

Because the large lipid droplets within adipocytes help with cellular structure, it is imperative to maintain the natural shape and structure of the cell when manipulating the

cell with fixatives and other chemicals. To do this, we used a formaldehyde / glutaraldehyde fixative in the presence of the fixative osmium tetroxide, a fixative for lipids. To determine if a longer fixing time is necessary with osmium tetroxide, we fixed adipocytes for either 30 min or 1 hr. There was no noticeable change in the size, shape or structure of the adipocytes, indicating that a longer fixation was not detrimental to the integrity of the cell (Fig 6). Because of this, the longer duration was chosen as ideal in order to allow for a more complete fixation of the lipids.

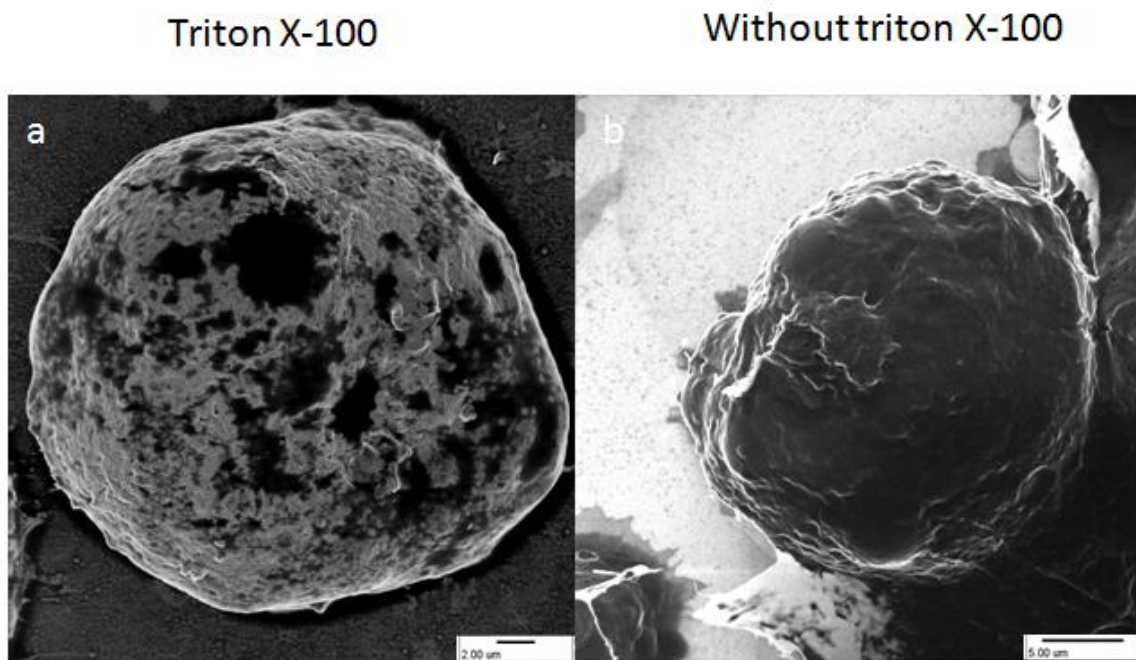


Figure 7. Effect of triton X-100 on 3T3-L1 adipocyte morphology, with or without triton X-100. Adipocytes were fixed overnight with 2% formaldehyde, 2.5% glutaraldehyde in 0.1M cacodylate buffer, 7.3 pH on Day 7 of differentiation. Next, cells were fixed in 4% osmium tetroxide for 1 hr. Following this, cells were either treated (A) with 0.1% triton X-100 for 15 min or (B) untreated. Finally, samples were dehydrated in acetonitrile overnight.

Optimization of duration for triton X-100 treatment of biological samples

To determine the effectiveness of a detergent, we next compared a sample with a triton X-100 treatment for 15 min with a sample that had no triton X-100. A visual difference in the morphology was present, with portions of the plasma membrane damaged for the triton X-100 treated cells (Fig 7).

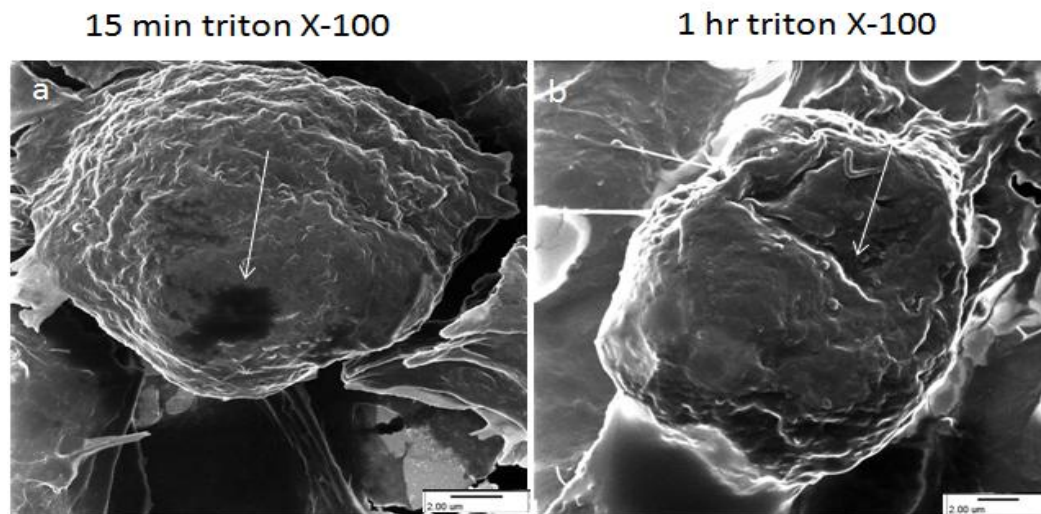


Figure 8. Comparison of treatment of triton X-100 duration. Day 7 adipocytes were treated with a fixative mixture of 2% formaldehyde, 2.5% glutaraldehyde in 7.3 pH 0.1M cacodylate buffer, 7.3 pH overnight, followed by 4% osmium tetroxide for 1 hr. Cells were then treated with 0.1% triton X-100 for (A) 15 min or (B) 1 hour. Following the permeabilization, cells were dehydrated in acetonitrile overnight. Arrows on the top portion of the images indicate effects of triton X-100 on cell structure. Arrows on the lower portion indicate individual cells following treatment.

We also tested for the length of time in which a triton X-100 treatment would completely destroyed the integrity of the plasma membrane or cell structure. At 15 min we saw visible holes along the plasma membrane, but at 1 hour we saw individual cells collapsing, as well as major damage occurring to many cells within a wider field of view (Fig 8).

30 min Osmium tetroxide,
10 min triton X-100

1 hour Osmium tetroxide,
10 min triton X-100

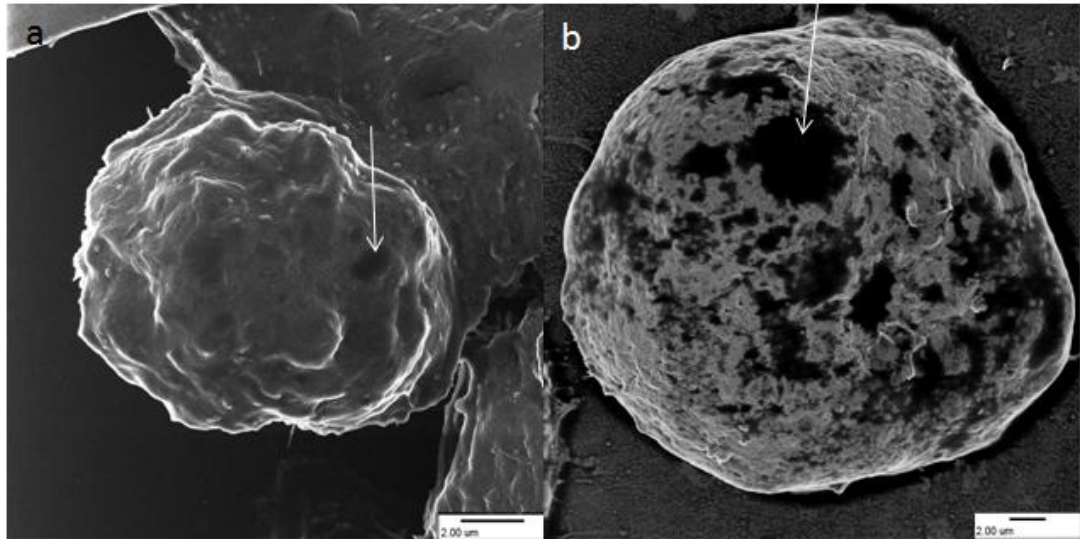


Figure 9. Osmium tetroxide fixation is more effective at 1 hr when adding triton X-100. Day 7 adipocytes were subjected to 2% formaldehyde, 2.5% glutaraldehyde in 0.1M cacodylate buffer, 7.3 pH overnight. Next, cells were fixed in 4% osmium tetroxide for 30 min or 1 hr. Following cells becoming permeabilized with 0.1% triton X-100 for 10 min, the cells were dehydrated in acetonitrile. **(A)** Adipocytes fixed in 4% osmium tetroxide for 30 min and **(B)** adipocytes fixed in 4% osmium tetroxide for 1 hour. Both are with triton X-100 treatments for 10 min. Arrows indicate holes created by triton X-100.

To determine if treating adipocytes with the detergent triton X-100 would adversely affect cells treated with osmium tetroxide, we treated adipocytes with triton X-100 for 10 min after adding the osmium tetroxide fixative for 30 min or 1 hr. With this procedure we visually saw a change in the appearance of the cells, with the 30 min treatment having a more fluid look to it. This was indicative that the lipids were not completely fixed by the osmium tetroxide yet. The 1 hr treatment, however, has a harsh

appearance with cell surface damage, but also seems to have been fixed more completely based on the shape of the cell (Fig 9).

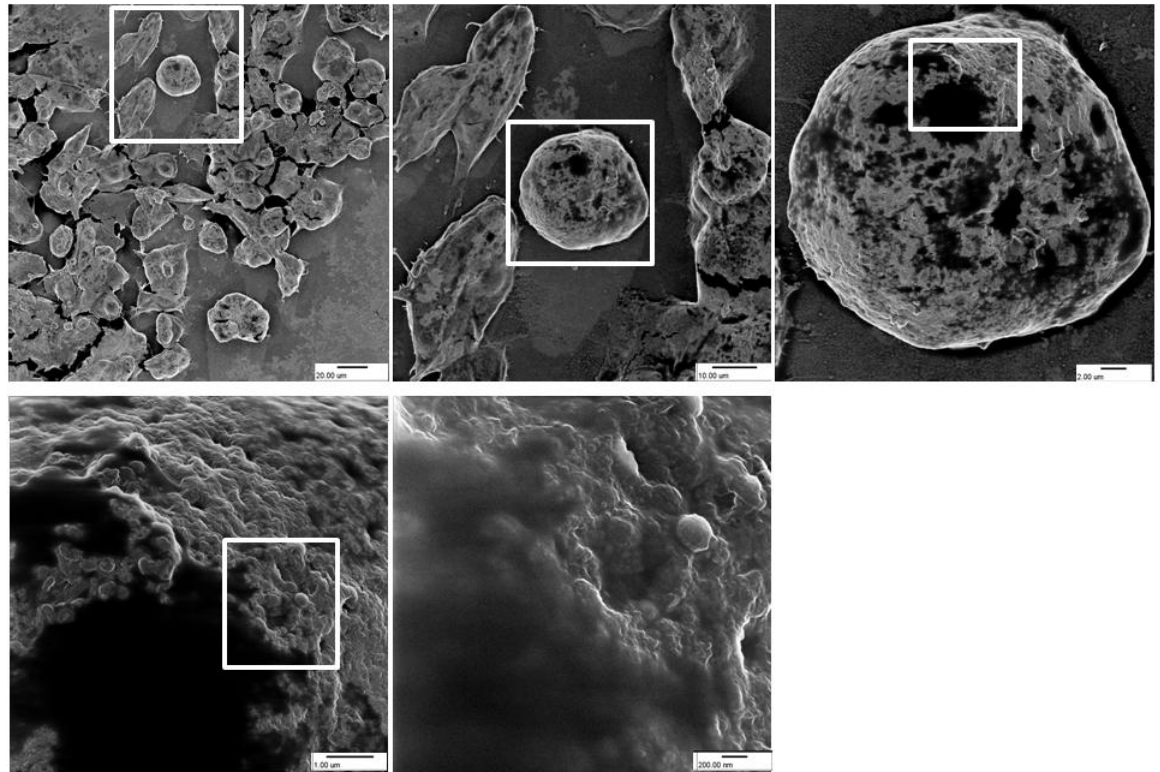


Figure 10. Series of magnifications of 3T3-L1 adipocytes. Day 7 adipocytes were serum starved for 4 hr. Cells were then fixed overnight with 2.5% glutaraldehyde and 2% formaldehyde 0.1M cacodylate buffer, pH 7.3, followed by a secondary 4% osmium tetroxide treatment for 1 hr. Cells were then subjected to 0.1% triton X-100 for 15 min, followed by a dehydration series in acetonitrile. White boxes indicate a magnified version of the area in following images.

Visualization of the plasma membrane of adipocytes with HIM

With parameters set for durations of fixatives and detergents, we next magnified the images to determine the level of detail that could be observed by HIM (Fig 10).

However, without the use of antibodies for marking structural proteins or receptors, we were not able to determine any specific proteins on the cell surface.

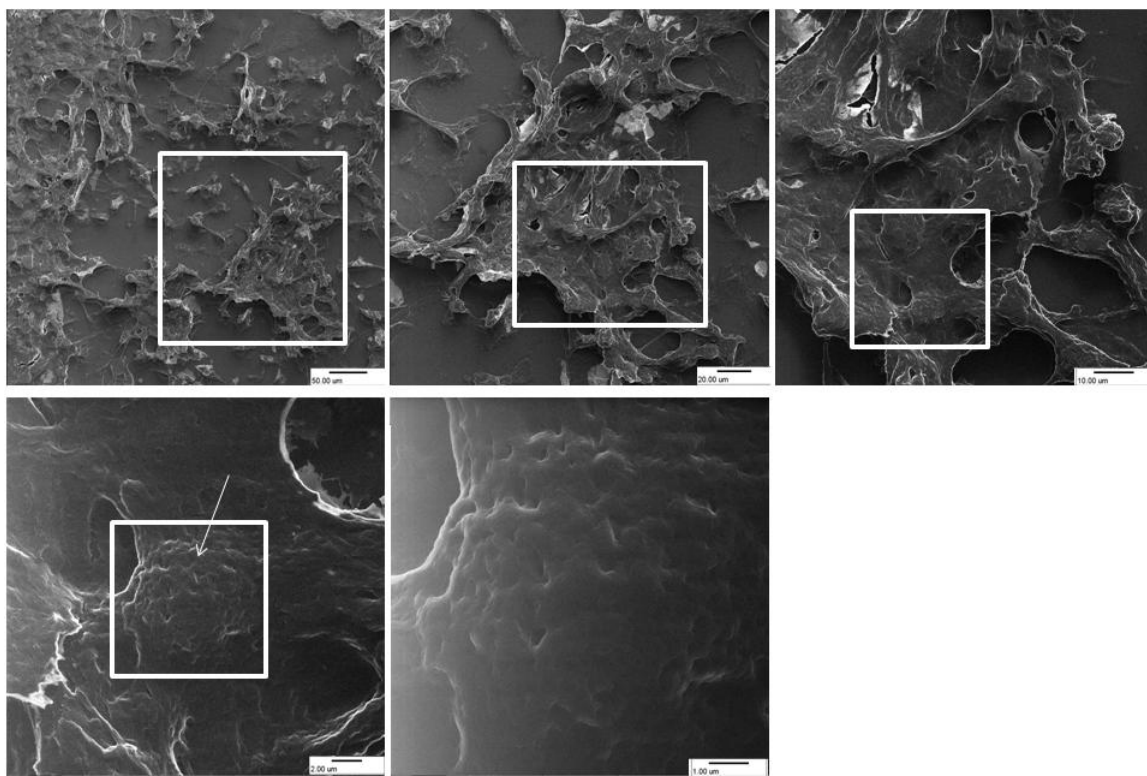


Figure 11. Plasma lawn assay of 3T3-L1 adipocytes. 3T3-L1 adipocytes were cultured on coverslips and were fixed overnight with 2% formaldehyde and 2.5% glutaraldehyde 0.1M cacodylate buffer, pH 7.3, rinsed in deionized water, and then incubated for 1 min in 0.5mg/ml poly-d-lysine. Following three rinses in hypotonic buffer, the cells were then sonicated at power 4-5. Following a dehydration series in acetonitrile, the coverslips were attached to HIM stubs via carbon tape. White boxes indicate the magnified portion of the field of view in subsequent images.

Visualization of the intracellular plasma membrane of adipocytes using a plasma lawn assay with HIM

We chose to determine if any structures on the intracellular portion of the plasma membrane could be analyzed using HIM. To establish a functional method for examining the intracellular surface of the plasma membrane, we performed a plasma lawn assay (described in Materials and Methods) with adipocytes Fig 11). From this we observed detailed images of intracellular structures present below the plasma membrane

at different magnifications (Fig 11). These studies demonstrate that the protocol we developed for preparing biological samples can be used for helium ion microscopy.

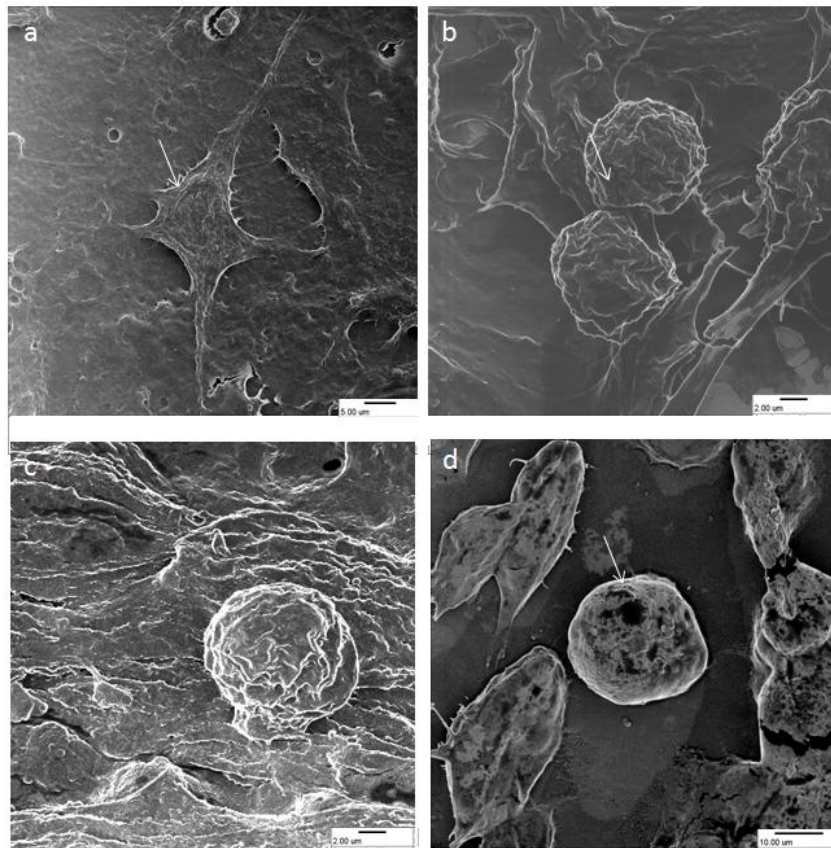


Figure 12. Days of differentiation of 3T3-L1 pre-adipocytes and adipocytes. 3T3-L1 adipocytes were cultured on gold plated coverslips and fixed with 2% formaldehyde and 2.5% glutaraldehyde in 0.1M cacodylate buffer, 7.3 pH for 1 hr, and 4% osmium tetroxide for 1 hr, treated in 0.1% triton X-100 for 15 min, and dehydrated in acetonitrile overnight at the time points of (A) Day 0, (B) Day 2, (C) Day 4 and (D) Day 7. Following the treatment with fixative, cells were rinsed with deionized water, dehydrated with acetonitrile, and viewed under the HIM. Arrows indicate individual cells for each day of differentiation.

Effectiveness of biological sample preparation on various cell types

To determine if our established protocol for biological sample preparation is effective for a range of cell types, we used adipocytes at various days of differentiation. Using the 3T3-L1 cell line was ideal because it has a range of morphologies, beginning with fibroblastic preadipocytes and ending in a spherical differentiated form by day 7 of differentiation. We tested the optimal conditions we determined previously for differentiated adipocytes on day 0, day 2, day 4, and day 7 adipocytes (Fig 12). Our results indicated that our protocols for cellular preparation for HIM are suited for fibroblastic cell types as well as the more spherical cell types such as adipocytes.

CHAPTER IV

DISCUSSION

Various myosin isoforms have been shown to be involved in vesicle trafficking , most notably in plants, but in other systems as well, such as in yeast, drosophila, and mouse adipocytes [4, 38]. Past studies have shown other potential functions for myosin proteins, including adhesion, exocytosis, cell movement, and protein docking and fusion with the plasma membrane [2, 27, 38]. A necessary role for both F-actin and myoIIA in GLUT4 translocation to and docking at the plasma membrane has also been demonstrated [4, 8]. However, myoIIA has been clearly described as having no role in the translocation of GLUT4 vesicles along actin tracks to the plasma membrane [3]. Instead, it has only been observed with its co-localization with GLUT4 at the plasma membrane suggesting that it may be involved in GLUT4 docking and fusion with the plasma membrane [4].

While myoIIA and F-actin are each individually described as important factors involved in insulin-stimulated glucose uptake in adipocytes, it is important to begin describing their roles as a myosin-actin complex. Studies have begun describing co-localization of GLUT4 and myoIIA or GLUT4 and actin [2, 8], but studies involving myoIIA and actin interaction in adipocytes during insulin stimulation have been limited [9, 26]. Because of the important cross-bridge contraction that actin and myosin establish

within muscle cells, we attempted to provide evidence that myoIIA and F-actin overlap cortically during insulin stimulation in adipocytes when GLUT4 docks and fuses with the plasma membrane. Also providing support for myoIIA and F-actin interaction is a study by Wilson et al. demonstrating that the secretion of insulin from β -cells of the pancreas requires cytoskeletal remodeling at the plasma membrane by myoIIA. However, this remodeling is described as a depolymerization event [29]. Our study is the first to provide evidence that myoIIA and F-actin at least co-localize at the plasma membrane, providing evidence for an interaction to occur during insulin stimulated glucose uptake. Taken together, either contraction or depolymerization of F-actin by myoIIA is suggested to occur in the presence of insulin to allow for GLUT4 fusion with the plasma membrane.

We specifically showed the role that calcium plays in co-localization of GLUT4, myoIIA, and F-actin at the plasma membrane during insulin stimulated glucose uptake. We used A23187 (a calcium ionophore) and BAPTA-AM (a calcium chelator) to alter levels of calcium in 3T3-L1 adipocytes. These experiments provided evidence of the necessity of calcium for myoIIA and F-actin co-localization at the plasma membrane during insulin stimulation (Figs 2 and 3). These studies also demonstrated the effectiveness of calcium as a regulator of myoIIA translocation without the need for insulin, due to its regulation of MLCK.

Furthermore, the co-localization of myoIIA and F-actin indicates a possible interaction between the two. The most likely interaction would be a contraction of the myosin-actin complex. However, studies have implicated myosin as a depolymerization agent for F-actin [8, 9], indicating that there are two possible ways that myosin can

interact with F-actin under insulin conditions. Further research into the type of interaction that occurs is necessary to better understand how GLUT4 docks and fuses with the plasma membrane during insulin stimulation of adipocytes.

We determined that calcium is necessary but not sufficient in the co-localization of GLUT4 and F-actin at the plasma membrane. However, calcium was sufficient for myoIIA translocation to the plasma membrane. Because the co-localization of all three proteins is necessary for GLUT4 fusion to occur [3, 4, 8], our studies suggest that calcium alone will not allow for GLUT4 docking and fusion at the plasma membrane to occur in 3T3-L1 adipocytes.

To gain further insight on alterations at the plasma membrane during insulin stimulated glucose uptake, we wanted to visualize the plasma membrane at high resolution using HIM. With very little information on how to prepare biological samples for HIM, we first had to develop a technique for preparing adipocytes for visualization under the HIM before we could do detailed analysis of the cell surface. In order to utilize the full potential of the HIM, the sample itself was prepared to address specific parameters related to absorbance of diffracting electrons. For example, glass coverslips allow many secondary electrons to diffract, causing poor resolution with the sample. To reduce this issue, substrates known to absorb electrons were tested. However, this is a multi-step process that includes not only the substrate, but the fixative, types of washes, detergents, and durations of each component as well. Because there are established protocols for similar microscopes, such as the SEM and gallium ion microscope, we had a starting point for our new protocol.

From the substrates we tested, gold coated coverslips were the optimal substrate for HIM preparation. Because the gold is transparent on a coverslip, it allowed for us to easily view the cells with a light microscope. The most important factor with using gold, though, is that it will absorb diffracting electrons during visualization under the HIM. This will in turn allow for a higher resolution.

Choosing to use both formaldehyde and glutaraldehyde as the fixative for cellular proteins was made to maintain the integrity of the shape and size, due to the limited cytoskeletal structure within adipocytes. This is mainly due to the large lipid droplets within adipocytes contributing to the structure. We therefore used osmium tetroxide to fix the lipids to stabilize the structure before performing a dehydration series in acetonitrile. Future studies can further manipulate the methods used in fixing the cells, such as using osmium tetroxide at the same time as formaldehyde and glutaraldehyde [32, 33]. Also, the dehydration series used could be compared to ethanol, or a combination of both could be used. A comparative study would determine if these combinations have any lasting effect on the structural integrity of the adipocyte. To protect from cellular structural damage, preceding the dehydration series the sample could be treated with uranyl acetate [32]. Because fixatives and alcohols are known to have an effect on the shape, size and conformation of cells and their structures, further studies with the HIM need to be done to show the change actually occurring. The wide range in differences can help in establishing a better protocol for future cell preparations with any type of high intensity microscope, not just the HIM.

The next experiment we performed using a plasma lawn assay was to determine if we could detect changes inside the plasma lawn to compare to the outside. Even subtle differences can give us a starting point with the use of antibodies to see target proteins. There is much potential with the use of gold labeled antibodies, with future studies having potential to establish the location and abundance of proteins not only on the plasma membrane, but intracellularly along the plasma membrane.

Finally, we looked at various days of differentiation to determine if our protocol for cellular preparation would be applicable with other cell types. Although fibroblasts do not have as much depth as differentiated adipocytes, the images for the different cell types were very descriptive at high resolution regardless of depth. This protocol is therefore useful for many applications, regardless of the cell type a researcher may be interested in. Combined with some form of this protocol, the HIM will be an important tool for structural studies in the near future due to the high image quality obtained for biological specimens.

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